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Article Title

Unravelling the global invasion routes of a worldwide invader, the red swamp crayfish (*Procambarus clarkii*)

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Keywords

Admixture, invasion hubs, invasion process, mitochondrial DNA, propagule pressure,

ii. Summary

1. Understanding how introduced species succeed and become widely distributed within non-native areas is critical to reduce the threats posed by them. Our goal was to reconstruct the main invasion routes and invasion dynamics of a global freshwater invader, the red swamp crayfish, *Procambarus clarkii*, through the analysis of its genetic variability in both native and invasive ranges.

2. We inferred invasion routes and population structure from the analysis of a fragment (608bp) of the mitochondrial marker COI from 1,062 individuals of *P. clarkii* in addition to 354 GenBank sequences, for a total of 122 populations (22 natives and 100 invaded). Genetic structure was assessed using analysis of molecular variance and non-metric multidimensional scaling analyses. We analysed haplotype frequencies for the genetic variability in each locality and region. The haplotype network was depicted by using PopART software.

3. A high haplotype diversity was found in the native range (Hd: 0.90), but also in some non-native areas, such as western United States (Hd: 0.80), areas of Mexico (Hd: 0.78) and some hotspots in Europe (e.g., southern Spain or Italy), suggesting a complex pattern of multiple introductions. We grouped all localities in five differentiated groups according to biogeographic origin: the native area, West Americas, East United States, Asia and Europe. Additionally, the identification of 15 haplotypes shared between at least two localities, the phylogenetic network estimation and indices of genetic differentiation among localities allowed us to identify a large genetic

admixture in the native range; the two independent invasion routes (i.e. westwards and eastwards) in US from the native range (Louisiana and Texas) with translocations within each area; a stepping-stone introduction from US to Japan (involving few individuals) themselves introduced to China afterwards; the entry of *P. clarkii* from Louisiana (US) into southern Spain and their multiple secondary introductions over Europe as well as other possible introductions in central Europe.

4. Our study emphasizes the need for unravelling the global invasion routes and the demographic processes underlying the introduction of exotic species (i.e., admixture, bridgehead invasion effect and propagule pressure) to control the spread of invasive species. Our findings highlight the value of genetic analyses to identify the geographic origin of source populations as well as the variability of invaded areas in order to reconstruct invasion dynamics and facilitate management of invasive species (e.g. through environmental DNA monitoring).

iii. Main Text

Introduction

Humans have transported species across biogeographical barriers and introduced them to new territories for millennia (Forcina et al., 2015), but large-distance movements of species have increased exponentially in recent decades (Hulme, 2009; Lenda et al., 2014) driving to an homogenization of biotas that has involved the break-down of long-established biogeographical barriers (Capinha et al., 2015). Those species that are transported by humans, released into new environments, able to survive, establish self-sustained populations, thrive, become abundant and spread geographically, are considered invasive species (Jeschke et al., 2014). Biological invasions today are perceived as major components of global change, with severe negative environmental (Simberloff et al., 2013; Blackburn et al., 2014; Jeschke et al., 2014) and socio-economic impacts (Vilà et al., 2010). To manage invasive species, it is of vital importance to identify invasion routes (De Kort et al., 2016). However, most knowledge about the transport routes of invasive species is

based on historical and observational data, which are usually scarce, confusing and sometimes inaccurate (Roman, 2006; Haydar, 2012). Population genetic studies provide valuable tools to identify areas of geographic origin of introductions, to detect single versus multiple introductions, and to describe expansion patterns (Lejeusne et al., 2014; Cristescu, 2015; Blakeslee et al., 2017; O'Hanlon et al., 2018; Fang et al., 2018), though caution must be used when interpreting demographic history over such short timescales (Fitzpatrick et al., 2012). Such information can be useful for the management of invasive species and for the prevention of future introductions (Estoup & Guillemaud, 2010), and phylogeographic studies have been proposed as an integral tool of biodiversity conservation planning (van de Crommenacker et al., 2015).

Many invasive species have high economic value, which often results in their deliberate introduction by humans into non-native areas where they can spread rapidly due to secondary introductions (see Audzijonyte et al., 2017; Cao et al., 2017; Huang et al., 2017). Unlike accidental introductions that are facilitated by humans, species that are deliberately introduced may have a higher chance of success because humans take action to ensure such success (Pyšek et al., 2011). For example, deliberate introductions often involve a high propagule pressure (i.e. number of introduction events and/or size of propagules; Lockwood et al., 2005; Simberloff, 2009), the genetic admixture of introduced populations (i.e. the mixing of populations from genetically distinct source populations; Dlugosch & Parker, 2008; Rius & Darling, 2014; Hufbauer, 2017), and the subsequent invasive bridgehead effect (i.e. a particularly successful invasive population serves as a source for new introductions, Lombaert et al., 2010; Estoup & Guillemaud, 2010).

Due to the high intensity of human disturbances and the high connectivity among inland water systems, freshwater environments are especially susceptible to invasions (Strayer, 2010; Havel et al., 2015; Tricarico et al., 2016). Also, many freshwater species can be harvested from the wild and/or cultivated in farms for commercial purposes, providing a high socioeconomic value (e.g.,

aquaculture; FAO, 2011). Among these, several invertebrates are valued either for human consumption or as food for other cultured animals (e.g. shrimp or prawn for fishes, crayfish for bullfrogs, etc.), which provides a high economic return (Resh & Rosenberg, 2015). Freshwater crayfish are favoured for farming since they do not have a larval phase and are polytrophic, they are relatively easy to rear compared with other cultured crustaceans (Holdich, 1993), and their consumption has a long-lasting tradition in many regions worldwide (Gherardi, 2011). The red swamp crayfish, *Procambarus clarkii* (Girard 1852), native to southern USA and northern Mexico, has been successfully introduced into all continents except Australia and Antarctica (Loureiro et al., 2015) mainly due to its economic value (Hobbs et al., 1989). Owing to its biological and ecological characteristics, this crayfish is considered one of the worst invasive species worldwide, causing serious damage to biodiversity (e.g., other crayfish species, fish, amphibians, macroinvertebrates and macrophytes) and to human infrastructure and ecosystem services (e.g., irrigation canals, water quality, rice crops, etc.) (Geiger et al., 2005; Twardochleb et al., 2013). *Procambarus clarkii* is one of the most economically valuable aquatic species to be farmed (Huner, 2002; Souty-Grosset et al., 2016), generating tens of billions of US dollars (USD) per year in the world (http://www.fao.org/fishery/culturedspecies/Procambarus_clarkii/en#tcNA0064).

The first introductions of *P. clarkii* out of its native area took place in the early 20th century, when it was taken to the Hawaiian Islands (1923), the Pacific drainages of USA (1924), Japan (1927) and China (1929), with different motivations including aquaculture, fishing activities and food for cultured American bullfrogs *Lithobates catesbeianus* (Holmes, 1924; Penn, 1954; Brasher et al., 2006). *Procambarus clarkii* was often able to spread rapidly, occupying the rivers and lakes of non-native areas (e.g. Riegel, 1959, for California; Yue et al., 2010, for China). In the mid-1960s, a batch of crayfish was sent to Uganda from Louisiana, then translocated to Kenya, and later to other African countries (Huner, 1977; Lowery & Mendes, 1977). Concurrently, it artificially spread out of its native area in Mexico, and then to Costa Rica, Puerto Rico, Venezuela, and the Dominican

Republic in the 1970s (Huner, 1977), eventually reaching Brazil in the mid-1980s (Huner, 1986). In Europe, it was deliberately and legally introduced into Spain (Badajoz and Seville in 1973 and 1974, respectively) from Louisiana (Habsburgo-Lorena, 1978; 1986). In only 45 years, *P. clarkii* has colonized many countries in Europe, being widely established in Spain, Portugal, France, Italy, Belgium, Netherlands, Germany and the United Kingdom (see Kouba et al., 2014 for the entire European distribution of this species).

To date, most genetic studies of *P. clarkii* have focused on genetic variability at a regional scale (e.g., Barbaresi et al., 2007; Torres & Álvarez, 2012; Quan et al., 2014; Yi et al., 2018; Almerão et al., 2018). Of these, very few studies have attempted to unveil the invasion routes, and when performed, they did so only at a regional scale. Hence, almost nothing is known about the population genetics and invasion routes of *P. clarkii* at a global scale. The general objective of this study is to provide a comprehensive overview of the global invasion history of *P. clarkii*. We included not only most of the non-native range of this species in the Northern Hemisphere, but also an exhaustive sampling of its native area in order to confirm the invasion sources and routes previously in the literature and detect previously unreported ones. Hence, our specific objectives were: 1) to describe the invasion dynamics of *P. clarkii* at continental and global scales, identifying the main invasion routes; and 2) to examine the genetic variability and population structure of *P. clarkii* in the native area and across the non-native range, with special focus on Europe, to reveal potentially unreported introductions not cited in the literature.

Methods

Sampling

We collected 1,062 specimens of *P. clarkii* from 72 localities: 15 native (States of Louisiana and Texas, USA) and 57 non-native localities distributed within the Northern Hemisphere (i.e., western

US, eastern US, Europe and Japan) (Table 1 and Fig. 1). Crayfish were individually preserved in 96% ethanol. Average sample size per locality was 14.7 ± 6.6 individuals (mean ± SE; range 2-21) (Table 1). We included in our dataset the information for 354 additional individuals from 7 native and 43 non-native localities that we obtained from data already published in previous studies (Genbank Accession numbers: AY701195; JF438001- JF438004; JN000898- JN000908; JX120103- JX120108) available from Taylor & Knouft (2006), Filipová et al. (2011), Torres & Álvarez (2012) and Li et al. (2012), respectively. Thus, a total of 1,416 individuals from 22 native and 100 introduced localities (Fig. 1a; Table 1) were used for this study. The sequences recently published by Almerão et al. (2018) were not added into our global analyses of this study because our sequences were larger than theirs. Even so, we compared their results in a subsequent analysis (see Supplementary Material, Table S1 for synonymous haplotypes).

DNA extraction and sequencing

Genomic DNA was extracted from muscle tissue (gill tissue at LEN, FOR and PER localities; see Table 1 for more details) using a modified DNA salt-extraction protocol (composition: NaCl 25 mM, Tris 12.5 mM (pH 8.0), EDTA 12.5 mM (pH 8.0), 31.5 µL SDS 10%) and proteinase K (Aljanabi & Marinez, 1997). Logistical support was provided by the Laboratorio de Ecología Molecular, Estación Biológica de Doñana, CSIC (LEM-EBD). A fragment of the mitochondrial gene coding for the cytochrome *c* oxidase subunit I (COI) gene was amplified using the primers LCO1490 and HCO2198 (Folmer et al., 1994). Amplifications were carried out in a 20 µl reaction volume, with 1-5 µl of genomic DNA, 2 µl of 10x buffer, 0.8 µl of $MgCl_2$ (50 mM), 0.16 µl dNTP (100 mM), 0.5 µl primer LCO 1490, 0.5 µl primer HCO 2198 and 0.12 µl TAQ polymerase (Bioline). Polymerase chain reaction (PCR) consisted of an initial denaturation step at 94ºC for 5 min, followed by 30 amplification cycles (94ºC for 1 min, 47ºC for 1 min and 72ºC for 1 min) and a final elongation step at 72ºC for 5 min. Sequencing was performed by Macrogen Europe Company.

Genetic analyses

Sequences were edited using the software Sequencher™ v4.9 (Gene Codes Corp., © 1991–2009, Ann Arbor, MI 48108). Nucleotide sequences were aligned using the algorithm CLUSTAL W implemented in BioEdit (Hall, 1999). No insertions nor deletions (indels) were found. A hierarchical series of tests based on the Bayesian Information Criterion (BIC) was applied to identify the most appropriate nucleotide substitution model among 88 models tested, as implemented in jModelTest 2 (Darriba et al., 2012). We used the nested model Tamura & Nei (1993) with 133 parameters, 1382.91 –lnL for onwards analyses. DnaSP 6.0 software was used to calculate the number of polymorphic sites (S), haplotype diversity (Hd), nucleotide diversity (π), and total number of synonymous and non-synonymous mutations, for which nucleotide sequences were translated into amino acid sequences using the *Drosophila* mitochondrial genetic code (Rozas et al., 2017). The haplotype network was inferred by the TCS method (Clement et al., 2000) implemented in PopART software (Leigh & Bryant, 2015).

Because of a smaller sampling size (1 or 2 individuals), ILL, LAf, FR1, FR2, FR3 and VAL localities were excluded from downstream analyses. Pairwise ϕ_{ST} (*Phi*$_{ST}$) and hierarchical analysis of molecular variance (AMOVA) were calculated using Arlequin 3.5 (Tamura & Nei, 1993; Excoffier & Lischer, 2010). To examine the genetic differentiation between any two of the populations, ϕ_{ST} calculations were calculated assuming gamma-distributed substitution rates using the Tamura and Nei model (Tamura & Nei, 1993) to compute a distance matrix and 10,000 bootstrap pseudo-replicates were used to estimate the standard error. The *p-values* were corrected for multiple comparisons using the false discovery rate (FDR) control according to the Benjamin and Hochberg (BH) correction method (Benjamin & Hochberg, 1995). To ascertain the genetic structure of populations, AMOVAs were performed based on 10,000 random permutations. Due to the large native range of *P. clarkii*, we classified native localities into five groups according to

natural (e.g. river catchments) and administrative (e.g. country or state frontiers) boundaries: Mexico, Texas, Louisiana east (east of the Atchafalaya River), Louisiana west (west of the Atchafalaya River), and Mississippi River upstream (upstream starting at Monroe, Memphis and north to Illinois). However, for all datasets, two different *a priori* hypotheses were tested: (a) native versus introduced localities, and (b) population grouping according to biogeographical distribution of this species into 5 zones: (1) native area, (2) West Americas which included all samples from the USA west of Texas, including California, Oregon and Washington State, plus all samples from Hawaii and invaded Central America, (3) East United States (from Louisiana to the Atlantic Ocean and Chicago), (4) Asia, and (5) Europe. Another *a priori* hypothesis was analysed exclusively for European populations to test whether there were one (i.e., the whole of Europe) or two genetic clusters within Europe (i.e., southern and northern areas of *P. clarkii* distribution). A dissimilarity matrix of Jost's D_{est} distances was also calculated with 10,000 replicates using SPADE (Jost, 2008; Chao & Shen, 2012). Based on ϕ_{ST} and D_{est} estimates, two non-metric multidimensional scaling (NMDS) analyses were used to graphically represent the differentiation among localities and their respective zones (described above) using the *vegan* package in R (Oksanen, 2013).

Results

Among the 1,416 specimens of *P. clarkii* analysed, we obtained a matrix of 608 base pairs (bp) of the cytochrome *c* oxidase subunit I (COI) with 54 polymorphic sites, yielding 65 haplotypes. Sequences of all haplotypes were submitted to GenBank and assigned Accession Numbers: MK026671 - MK026735. Most of the nucleotide substitutions were synonymous, but four non-synonymous changes were identified (2 substitutions at the 1st position corresponding to a change from an Isoleucine to a Valine, and from a Methionine to a Valine, respectively; and 2 substitutions at the 2nd position corresponding to a change from a Valine to an Alanine, and from a Threonine to a Methionine, respectively). Of these four non-synonymous changes, one singleton was found in the

ALB locality (Spain) and three parsimony sites were located at SMA and COM localities in Texas (US) and in the DU locality in the invaded area of Mexico (see abbreviations in Table 1 and marked in haplotype network in Fig. 2).

The overall haplotype diversity (Hd) and nucleotide diversity (π) were 0.76 and 0.0040, respectively. The native area showed the highest haplotype and nucleotide diversity (0.90 and 0.0055, respectively), with the highest figures being found in WOO, DES, MON and PIE localities (Table 1). For invaded areas, haplotype and nucleotide diversities varied considerably between regions: Hd: 0.80 and π: 0.0048 in non-native US, Hd: 0.78 and π: 0.0056 in non-native Mexico, Hd: 0.46 and π: 0.0023 in Asia and Hd: 0.58 and π: 0.0022 in Europe, respectively (for localities see Table 1). Of the entire dataset, a total of 15 haplotypes were shared between at least two sampling localities (Fig. 1), of which Hap_04 was present at high frequency almost worldwide, irrespective of the native (up to 13 localities) or non-native (up to 76 localities) status of the populations. Other haplotypes were shared between continents, including coincidences between US and Europe (Hap_01, Hap_03, Hap_05, Hap_09 and Hap_29) and North America and Asia (Hap_02 found in invaded localities in Mexico, California, Japan and China, and Hap_40 shared between the native area and Japan). Conversely, 50 haplotypes were restricted to one locality (i.e., private haplotypes), 29 of them found in the native area and 21 being exclusive to one of the invaded localities (see Supplementary Material, Table S2).

The statistical parsimony haplotype network showed a star-like structuring centered around the Hap_04, which appeared in almost half of sampled crayfish (618 specimens) geographically widely distributed over all zones (13 native and 62 invaded localities) (Fig. 2 and see Supplementary Material, Table S2). Moreover, there were other smaller star-like structuring around three haplotypes (Hap_01, Hap_20 and Hao_09). Hap_01 was mainly distributed in the US, both in its native (9 localities in Louisiana) and non-native range (TOP and PIN in western US; and PER, LEN, FOR and CHI in Atlantic area), but also was found in two Spanish localities (ECO and GIJ).

Hap_20 was found in Louisiana and across the western US. In addition, it closely joined (1 mutation) to Hap_28 which is widely found in Asia. Hap_09 was broadly distributed among the native localities in Louisiana, as well as in three invaded North American localities, and two Spanish and one French locality (AR4, CHO and FR1, respectively). In addition, this central haplotype was connected by only one mutation with Hap_40 which was present in Japan. A thorough analysis of the haplotype network in the native range (see Supplementary Material, Fig. S1) showed no clear genetic structure except for Texas localities (Hap_15-19 and Hap_48-50), Mexico localities (Hap_61-62) and the Hap_20 which was mainly found in southeastern Louisiana. Additionally, Hap_04, Hap_01 and Hap_09 were widely distributed over all localities in the native range as well as many localities grouped evolutionarily differentiated haplotypes (e.g., MON, NAT or LO localities), indicating a large genetic admixture in Louisiana.

Due to the large native area of *P. clarkii*, we tested whether the native area clustered into 5 groups (Mexico, Texas, Mississippi River upstream, Louisiana east, and Louisiana west). As haplotype network showed, AMOVA also revealed a slight genetic structure within the native area, where a small fraction of the total variance was due to between-group variance (29.0%); nevertheless, most of the variance was explained by variation within populations (61.6%) (Table 2). This may be due to the high proportion of private haplotypes (Fig. 1b and Fig. 1c). For the whole dataset, native and invaded areas worldwide were not clustered into two different genetic groups because only 14.3 % of total variation was due to differences between groups (Table 3), indicating the high variability of *P. clarkii* in the invaded range. However, after classifying the whole set of localities according to their biogeographical ranges (see Methods), 39.6% of total variation was still explained by differences within localities, but 36.0% of total variance was due to differences among these 5 established zones (Table 3). This result seems to indicate a slight genetic structure among these zones. In Europe, a moderate genetic structure was detected between the northern and southern distribution areas of *P. clarkii* (Fig. 1g), where Hap_04 was predominant in South Europe and

Hap_11 in Central Europe. AMOVA analyses showed that 40.7 % of explained variance was due to differences between both genetic clusters (Table 4).

In a NMDS plot based on D_{est} distances (Fig. 3), most localities remained within the 95% confidence intervals (CI) of the native area group, except some localities from Japan, China and western North America, in which Hap_28 was present at high frequency. This is due to the fact that we did not find this haplotype in the native area, despite the exhaustive sampling done there. All localities from eastern US were closely grouped within the range of the native area. However, the localities from western North America were more different from each other, for instance PIN was closer to European localities, whereas VEN and CH were more similar to Asian locations. In addition, the other locality from western North America not only had a clear proximity to each other but also to localities from within the native area (COM, SMA, COC and NL), indicating a similarity among them. The result of AMOVA analysis for European populations, where two genetic clusters were found between localities from the northern and southern European distributions, was also reinforced by NMDS plot. LON, HOL, ECA, BIO and BRI localities (depicted by a green triangle in the NMDS plot) were situated all together outside the 95% CIs of the European group, having greater proximity to Texas and Mexico localities from the native area and those from western America than to south European localities.

Discussion

The high haplotype diversity of *P. clarkii* found in some invaded localities suggests that its global invasion, driven mostly by human-mediated introductions, may have involved admixture in the native range, an invasive bridgehead effect, and high propagule pressure. However, we also detected low levels of genetic diversity in some non-native areas (e.g. Asia), attributable to potential bottlenecks or founder effects. Our results allow the identification of the likely geographic origin

and main routes of invasion, helping us to understand how the invasion has happened over a long time scale (Fig. 4).

The native range of the red swamp crayfish

Admixture has been proposed to be a causal mechanism triggering the invasiveness of some introduced species (Kolbe et al., 2007; van Boheemen et al, 2017; Fischer et al, 2017; Wagner et al., 2017; but see also, Rius & Darling, 2014) by enhancing genetic variability, thus improving population growth, decreasing the risk of extinction, and favouring adaptation to novel environments. In the present study, we found the highest haplotype diversity in the native area. The vast majority of haplotypes found in invaded areas also appeared in Louisiana but not in other native populations of US or Mexico. This pattern is arguably related to the commercial exploitation of this species in Louisiana, where *P. clarkii* has been reared, harvested and sold globally by food-industry companies for a long time (Gary, 1975; Alford et al., 2017). Although some genetic clusters seem to be differentiated between east and west Louisiana (Hap_01, Hap_04 and Hap_20 predominated in Louisiana east while Hap_03 and Hap_08 predominated in Louisiana west), Texas and Mexico localities, most of the genetic variation in those areas occurred within localities. The lack of a clear genetic structure in the native area might imply a pattern of admixture owing to farming activities in Louisiana, in which crayfish are often exchanged and translocated from wild to captive populations (Huner, 2002). Similar genetic patterns of native admixed populations have been identified in other species related to aquaculture such as the topmouth gudgeon, *Pseudorasbora parva*, when they were translocated together with Chinese carp species (Hardouin et al., 2018). The exchanges found in the Louisiana populations do not seem to have occurred in Texas, since eight private haplotypes were found in two populations and almost all haplotypes were grouped together (see Hap_15-19 and Hap_48-50). Admixed source populations, like for *P. clarkii* in Louisiana, can lead to high genetic variability in invasive populations, thus allowing the invasive species to face novel environments and to thereby increase invasion success in the introduced range.

But this assumption should be interpreted with caution since some species are able to show high invasiveness despite low genetic variability, such as *Procambarus virginalis*, a potentially highly invasive parthenogenetic crayfish that is able to establish wild populations from a single released individual (Feria & Faulkes, 2011).

The invasion dynamics of the red swamp crayfish

According to the literature, the first invasion of *P. clarkii* took place in Hawaiian streams in 1923 (Brasher et al., 2006) and in California in 1924 (Holmes, 1924). However, the geographical origins of both invasions remain unclear. In 1934, another event of introduction occurred in the island of Oahu, Hawaii, from Santa Barbara, California, from which subsequently *P. clarkii* apparently spread over the rest of the Hawaii archipelago (Penn, 1954). The Hap_27 found in each of these US states (California and Hawaii) may confirm this second introduction event of *P. clarkii* from California, but this result should be treated with caution since only four crayfish were sampled from Hawaii. In the continental USA, the California introduction was followed by later introductions to Oregon in the early 1980s (Larson & Olden, 2011) and to Washington State in the 2000s (Mueller, 2001). Theoretically, we might expect higher genetic variability in California, with a decrease of variability from the place of first introduction (California) northwards (Washington, Oregon) due to secondary bottlenecks and/or founder effects. Our results seem to indicate a more complex pattern of invasion (Fig. 4), in which shared haplotypes between populations in the western US confirm the connectivity among localities (Hap_01, Hap_09, Hap_20 or Hap_27). The development of the crayfish industry in California, where *P. clarkii* has been cultured and traded for many years, seems to have contributed to the dispersal of the crayfish along the West Coast of the US (Comeaux, 1978; Mueller, 2001). Moreover, this scenario might have been favoured by the large number of biological supply companies in this area, and also by the use of live animals for classroom observations, some of which were given to students after school-years to take home and were probably released in the wild later on (Larson & Olden, 2008).

Regarding the origin of California populations, we were not able to identify the precise geographic origin of this invasion because though low ϕ_{ST} values were found among California and native localities, and these native localities were not close to each other (see Supplementary Material, Table S3). The haplotypes found in Topanga Creek (TOP) suggest that the origin of this invasion came from southeastern Louisiana, but the presence of the Hap_08 (mainly distributed in western Louisiana; and considerably distinct from ancestral haplotype), would contradict this idea. For the other two California localities (VEN and SYZ), we were also unable to unveil their origins accurately because their haplotypes were not shared with the native area; however, their private haplotypes were more evolutionarily related to Hap_04 and Hap_20, which would indicate again a possible origin from southeastern Louisiana. Given that crayfish populations in Topanga Creek were recently established (around 2001, Garcia et al., 2015), this population could come from previous established populations in California, having undergone a possible bottleneck. If so, we could be underestimating the haplotype diversity in the area and more haplotypes would be present in California. This latter surmise is reinforced by the fact that *P. clarkii* has long been considered a pest in southern California (Riegel, 1959), the higher haplotype diversity in population WAV (Oregon) and the presence of Hap_04 at a high frequency only in PIN (Washington State). This suggests that (1) more genetic variability is to be expected in California, acting as an admixed or bridgehead zone because of its anteriority in introduction and the numerous biological supply companies which can move live crayfish, or (2) other distinct introduction events may have occurred in northern states from the native area, which seem unlikely given the great demand for the crayfish industry in California (Comeaux, 1978) and since the northwest US is the native range of another commercially and culturally important crayfish species (i.e., signal crayfish, *Pacifastacus leniusculus*) (Holdich, 1993).

Localities from the eastern US showed a different haplotype frequency (Hap_01 was the most common haplotype) from those of the western part of the country, suggesting another independent route of invasion to North Carolina and north of Illinois with subsequent secondary events (Fig. 4). This pattern is congruent with the native area located in the middle of the US, making it easier to move crayfish in two independent directions than from coast to coast, as well as the presence of one of the biggest biological supply companies in North Carolina (US) which was supplying most of the eastern US invaded areas. Although the eastern US is a suitable area for *P. clarkii* (Larson & Olden, 2011), the low haplotype diversity found in eastern localities of the US (from Hd = 0.20 in LEN to Hd = 0.50 in FOR) suggests a low propagule pressure from the native area or from shipments of the biological supply company in North Carolina.

In Asia, according to the literature (Penn, 1954), 100 specimens of *P. clarkii* were carried from New Orleans to Japan in 1927, of which only 20 specimens arrived alive to a pond near Tokyo (Penn, 1954; Kawai & Kobayashi, 2005); two years later, *P. clarkii* from Japan were translocated to Nanjing, in China (Li et al., 2007). This historical report perfectly matches the genetic pattern (i.e., founder effect and strong bottleneck) found in Japanese and, overall, Chinese populations of *P. clarkii* (Yue et al., 2010; Li et al., 2012; Zhu et al., 2013, this study), in which a smaller batch was introduced to Japan and subsequent invasions came from the Japan population with few founders (Fig. 4). The lack of ectoparasites of the order Branchiobdellida is often attributed to long shipments in poor conditions (Gelder & Williams, 2015; Clavero et al., 2016). Kawai & Kobayashi (2005) found no Branchiobdellida on Japanese specimens of *P. clarkii*, a pattern that could support the hypothesis that all specimens of *P. clarkii* in Japan (and thus, in China) descend from the initial introduction at the end of 1920s. Our results show low haplotype diversity in Japanese and Chinese populations (Hd = 0.48 and 0.35, respectively) with only four haplotypes appearing in the extensive area sampled, only two of them at high frequency (Hap_04 and Hap_28), as similarly found by Li et al. (2012). Apart from Asian populations, Hap_28 was only found in few individuals of the CH

population (Mexico) and VEN (California) but not in the native area. Surprisingly, our genetic results seem to contradict previous literature because neither Hap_28 nor Hap_40 appeared in localities sampled around the native locations of New Orleans, Louisiana. The finding of Hap_28 in California and the similar date of both introductions (i.e., California in 1924 and Japan in 1927) suggests that a route of invasion from California to Japan is more plausible (Fig. 4). Additionally, as Hap_28 was a rare haplotype in our sampling (only 5 of the 988 non-Asian individuals carrying this haplotype), another old introduction into Asia seems unlikely because a different haplotype frequency would be expected. This strong genetic bottleneck did not prevent *P. clarkii* from invading successfully (Estoup et al., 2016) and becoming a pest across Japanese and Chinese territories (Penn, 1954; Kawai, 2017). A similar pattern has been recorded for the parthenogenetic crayfish, *P. virginalis*, in other areas (Feria & Faulkes, 2011). Finally, the presence of the Hap_40 in TOK (Japan) and NAT (northwest Louisiana) led to two possible hypotheses: (1) this haplotype was present in the initial translocated batch but has been lost in subsequent secondary invasions by genetic drift or bottleneck, or (2) one new introduction event has recently occurred from the native area but has not been spread yet (nor been reported). Of both hypotheses, the first one seems more plausible, but we are not able to resolve them.

Of all invaded areas, the European invasion by *P. clarkii* has perhaps been the best reported, with the first two events of introduction from Louisiana to Spain (Halsburgo-Lorena, 1978) and later into other European countries (i.e., in Spain Gutiérrez-Yurrita, et al., 1999; in France Changeux, 2003 and Laurent, 1997; in Italy Gherardi et al., 1999) (Fig. 4). The invasion routes through European countries and connectivity between European populations are poorly understood, possibly because they are due to multiple and uncontrolled deliberate introductions by private citizens (Clavero, 2016; this likely also occurred with signal crayfish, *P. leniusculus*, see Petrusek et al., 2017). In European populations, we found a moderately high overall haplotype diversity (Hd = 0.58; i.e. lower than for invasive US populations, but higher than in Asia). The European invasion has

probably not been based on as many introduction events as invasive American populations (e.g., California) given the differences in proximity to the native area (a possible cause of the higher haplotype diversity found on the American continent); however, the large number of *P. clarkii* imported to Spain (100 kg, around 6,500 crayfish) probably also included high genetic variability from the native area compared to the Asian introduction. According to our results, a clear decrease in haplotype diversity was found from the initial sites of introduction (Hd = 0.66 in rice fields near Seville, and Hd = 0.72 in Doñana National Park) northwards, excepted for TOS in Italy (Hd = 0.72), which could be explained by intensive farming activities on Lake Massaciuccoli (Gherardi et al., 1999) or a second introduction.

The most surprising result was the finding of two independent genetic groups in Europe. The Hap_04 was widely distributed over the Iberian Peninsula, South France and Italy, while the Hap_11 predominated in Northern France and Italy, Belgium, the Netherlands and United Kingdom, but was not found in the Iberian Peninsula. Two possible scenarios could explain this result: (1) we did not capture all haplotypes from the first introduction in southern Europe, and northern populations have undergone a strong bottleneck; or (2) another unreported introduction from outside Europe has occurred, independently from those reported from southern Spain (Fig. 4). The first scenario is unlikely, due to the extensive sampling effort on both the Iberian Peninsula and the native area. In such a scenario, the Hap_11 should have appeared in the Iberian Peninsula because of other high frequencies in North Europe. Moreover, Almerão et al. (2018) found nine haplotypes in Central France, four of which seem to match with our database but not Hap_11. On the other hand, unreported introductions of *P. clarkii* could be a consequence of the sales in pet shops which are common and one of the primary introduction pathways in Central Europe (Chucholl, 2015; Faulkes, 2015). These results, however, also support previous historical reports (Laurent, 1990; Holdich, 2002) suggesting how live *P. clarkii* may have been brought from Kenya to Europe in the 1970s. Both hypotheses could explain the presence of this haplotype across the

464 northern European range of *P. clarkii*. To clarify our results, samples from pet shops or African
465 samples of *P. clarkii* should be obtained in order to resolve the likely second invasion route. The
466 second scenario therefore seems the most plausible (as Barbaresi et al., 2007, also suggested), with
467 a plausible introduction from Kenya to Central Europe.

468

469 National and international translocations have occurred within Europe (Fig. 4). On the one hand, we
470 found Hap_04 and Hap_05 to be highly frequent all over the Iberian Peninsula to South France,
471 which perfectly matches with the literature signaling where live specimens having been translocated
472 from South Spain (Laurent, 1997). On the other hand, the presence of the Hap_06 at higher
473 frequencies in southern Portugal and dated reports of introduction events across Portugal seem to
474 confirm the spread of *P. clarkii* from south (near the first introduction site in 1973; Cruz & Rebelo,
475 2007) to north Portugal (Gutiérrez-Yurrita et al., 1999). In addition, Hap_06 was also found in
476 MAD (Spain) and LAZ (Italy), suggesting a connection among these invaded areas as well as POR
477 in Portugal and LEZ in the Ebro Basin, Northern Spain (Fig. 4). Another possible connection was
478 between TOS in Italy and southern Spain, with most haplotypes shared, suggesting another possible
479 invasion route. Continuous exchanges and secondary translocations of *P. clarkii* through invaded
480 areas have produced a very complex invasion process, which could accelerate the invasiveness of
481 this kind of species (Wagner et al., 2017).

482

483 Our results provide a clear example of how different features of introduction events and invasion
484 processes (e.g. genetic admixture, propagule pressure or secondary introductions) can generate
485 contrasting genetic diversity patterns across non-native populations of a global invader (Roman &
486 Darling, 2007). For example, Asian populations of *P. clarkii* underwent a strong bottleneck as a
487 consequence of the introduction of few individuals in a single introduction event, which arguably
488 originated from an already introduced population (probably in western US) that might have already
489 gone through previous bottlenecks. Genetic diversity was notably higher in the *P. clarkii*

490 populations in western US, probably due to the existence of numerous introduction events (e.g.
491 facilitated by vicinity to the native range and the development of biological supply companies),
492 involving large batches of individuals with high genetic admixture. The European case is apparently
493 intermediate, with numerous individuals imported from an admixed native range to SW Spain, from
494 which the species expanded across the continent through multiple secondary introductions,
495 involving a clear loss in haplotype diversity. However, higher genetic variabilities were found in
496 European (Petrusek et al., 2017) and Japanese populations of *P. leniusculus* (Usio et al., 2016) in
497 comparison to that reported by us for *P. clarkii*, arguably due to the combination of several
498 introduction events involving large batches of individuals and coming from a variety of origins in
499 the US, including native and non-native populations. A striking pattern deriving from our results is
500 that the invasiveness of *P. clarkii* does not seem to depend, at least in the short- and mid-term, on
501 the genetic diversity of introduced populations. Although genetic diversity can fuel invasiveness by
502 allowing the efficient adaptation of introduced populations to spatial and temporal variability in the
503 recipient ecosystems, the relationship between those two features is obscure (Estoup et al., 2016).
504 There is growing evidence that the loss of genetic diversity in introduced populations can be
505 compensated through epigenetic processes (Estoup et al., 2016). The most extreme example of high
506 invasiveness with low genetic variability is the clonal species *P. virginalis* (Feria & Faulkes, 2011),
507 which is able to thrive in a wide variability of environmental conditions (Andriantsoa et al., 2019).

508

509 Apart from informing about invasion routes, our results might also be relevant for new approaches
510 for the detection and surveillance of invasive species. Environmental DNA (eDNA) is a rapidly
511 emerging monitoring tool for freshwater invasive species based on the persistence of DNA
512 fragments in the environment (Ficetola et al., 2008; Mauvisseau et al., 2018). Large-scale
513 phylogeographic studies provide accurate datasets for improving invasive species detection
514 protocols based on eDNA (Ficetola et al., 2008; Larson et al., 2017). Admixture in both native and
515 invasive ranges, as well as the bridgehead invasive effect, has led to large intraspecific genetic

variability within and among invaded areas, which may reduce the efficacy of eDNA protocols (Wilcox et al., 2015). In fact, the spatial gradients in genetic variability and the presence of different genetic clusters in Europe reported here, probably led to the failure of eDNA probes in detecting French populations of *P. clarkii* (Tréguier et al., 2014; Mauvisseau et al., 2018), which had worked well with the less variable Chinese populations (Cai et al., 2017). Our study may thus be useful for the development of better site-specific eDNA-based protocols to detect *P. clarkii* (Manfrin et al., 2019).

Conclusions

Our results illustrate extensive admixture of *P. clarkii* in its native area, report two independent invasion routes in the US (i.e., westwards and eastwards), and support the historical reports of a single introduction event into Asia involving few individuals. They also suggest that Europe may have received *P. clarkii* through more introduction routes than the frequently reported imports into Spain. To find other likely introduction routes, more effort should be put on sampling in previously unstudied sites (e.g., Texas, pet shops, biological supply trade and/or Southern Hemisphere countries where other introduced populations might act as sources of invasion, for example, African or South American populations).

We have traced the complex scheme of invasion of *P. clarkii* (Fig. 4), with a key role for human-mediated dispersal. The economic value of *P. clarkii* and the ease with which it is transported have favoured the spread of the species worldwide (largely for aquaculture, the aquarium trade and other forms of human exploitation as food) as the consequence of multiple subsequent introduction events. Genetic admixture, invasive bridgehead effects, extensive genetic variation in the native area and high propagule pressure are apparent drivers of genetic variability across its broad geographic distribution. Such extensive genetic variability in invaded areas should be taken into account to improve management measures based on mtDNA for environmental detection of this

invasive species. Overall, invasive species, and invasive crayfish in particular, continue to be artificially introduced into more countries through the aquarium trade (e.g. fish species, Strecker et al., 2011; crayfish species, Patoka et al., 2014 and particularly *P. virginalis*, Faulkes 2015). The example of the successful worldwide invasion of *P. clarkii* highlights the high spread potential of intentionally introduced freshwater species, especially those species also involved in aquaculture (Naylor et al., 2001). Once a species has been introduced in a new territory, management strategies aimed at reducing the spread and impacts of invasive species should focus on avoiding secondary introductions and would benefit from the early detection of potential invasion hubs.

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Conflicts of Interest

The authors declare no conflict of interest.

v. References

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vi. Tables

Table 1. Genetic diversity parameters based on the COI gene for each *Procambarus clarkii* locality. Note that localities are grouped in biogeographical zones (see Methods). Sequences retrieved from GenBank are shown in italics (see references in Materials and Methods section). *: 0.05 > p > 0.01; **: 0.01 > p > 0.001; ***: p < 0.001

Country	Locality	Code	Lon	Lat	N	*h*	Hd	*π*	R2	Tajima	Fs
NATIVE RANGE					179	39	0.902	0.00549			
East Louisiana											
	Poison	LA2	30.220	-91.614	20	5	0.795	0.00349	0.172	0.810	0.854
	Haha Bay	LA4	30.147	-91.628	20	8	0.775	0.00395	0.104	-0.519	-1.573
	Baton Rouge	BAT	30.370	-91.189	4	3	0.833	0.00302	0.265	1.090	0.006
	Morgan City	MOR	29.767	-91.127	10	6	0.867	0.00428	0.157	0.215	-1.164
	Pierre Part	PIE	29.950	-91.283	10	8	0.956	0.00450	0.135	-0.144	-3.882**
	Des Allemands	DES	29.798	-90.505	4	4	1.000	0.00630	0.114*	0.039	-0.884
	Jean Lafitte	JEA	29.732	-90.075	9	4	0.694	0.00265	0.172	-0.526	-0.061
	New Orleans	*LAf*	*29.950*	*-90.083*	*1*	*1*					
West Louisiana											
	Abbeville	ABB	29.911	-92.200	4	3	0.833	0.00247	0.276	-0.754	-0.288
	Alexandria	ALE	31.097	-92.493	4	3	0.833	0.00877	0.327	-0.222	1.606
	Woodworth	WOO	31.186	-92.467	4	4	1.000	0.00356	0.223	-0.065	-1.741*
	Natchitoches	NAT	31.740	-93.077	17	6	0.765	0.00816	0.199	1.495	2.133
	Kaplan	*LAt*	*29.991*	*-92.260*	*5*	*2*	*0.600*	*0.00197*	0.300	1.459	1.688
	Calcasieu L.	*LO*	*29.870*	*-93.260*	*10*	*4*	*0.533*	*0.00643*	0.143	-0.352	2.256
Upstream Mississippi River											
	Monroe	MON	32.497	-91.669	5	4	0.900	0.00724	0.218	0.132	0.286
	Memphis	MEM	35.366	-90.033	5	2	0.400	0.00066	0.400	-0.817	0.090
	Horseshoe	*ILL*	*37.138*	*-89.343*	*1*	*1*					
Texas											
	Comal	COM	29.711	-98.134	18	6	0.686	0.00170	0.103	-0.917	-2.350*
	San Marcos	SMA	29.882	-97.934	14	3	0.560	0.00103	0.157	-0.011	-0.072
Mexico											
	Sabinas Hidalgo	*NL*	*26.483*	*-100.221*	*4*	*1*					
	Río Jiménez	*CON*	*29.154*	*-100.764*	*5*	*2*	*0.400*	*0.00066*	0.400	-0.817	0.090
	Río Sabinas	*COC*	*27.969*	*-101.582*	*5*	*1*					
WEST AMERICAS											
Non-native US											
	Santa Ynez	SYZ	34.557	-119.881	10	3	0.378	0.00190	0.187	-1.388”	0.762
	Topanga	TOP	34.064	-118.587	20	5	0.679	0.00859	0.194	1.541	4.185
	Ventura	VEN	34.345	-119.299	4	2	0.667	0.00439	0.333	2.080	2.719

	Pine	PIN	47.587	-122.044	21	3	0.581	0.00183	0.186	0.883	1.537
	Waverly	WAV	44.640	-123.069	20	7	0.832	0.00501	0.121	-0.356	0.057
	Waiau	HW	19.713	-155.149	3	1					
Non-native	Mexico				13	4	0.782	0.00557			
	Teopisca	*CHIS*	*16.554*	*-92.476*	*5*	*1*					
	El Arenal	*DU*	*24.043*	*-104.428*	*5*	*2*	*0.600*	*0.00493*	0.300	1.686	3.526
	Las Varas	*CHt*	*29.797*	*-106.693*	*3*	*1*					
Costa Rica	*Cachí Dam*	*CR*	*9.825*	*-83.821*	*4*	*2*	*0.500*	*0.00082*	0.433	-0.612	0.172
EAST USA											
	Pee Dee	FOR	36.150	-80.291	4	2	0.500	0.00082	0.433	-0.612	0.172
	Pamplico	LEN	35.244	-77.559	10	2	0.200	0.00033	0.300	-1.112	-0.339
	Albemarle	PER	36.268	-76.378	21	3	0.338	0.00100	0.111	-0.707	0.204
	North Shore	CHI	42.032	-87.710	20	5	0.442	0.00113	0.105	-1.888*	-2.091*
EUROPE											
Spain					355	13	0.469	0.00239			
	Balboa	EXT	38.883	-6.871	20	2	0.395	0.00065	0.197	0.723	0.976
	Manecorro	MAN	37.124	-6.489	20	5	0.716	0.00443	0.143	0.214	1.571
	Cantaritas	AR4	37.046	-6.213	20	5	0.663	0.00447	0.140	0.243	1.596
	Colomera	GRA	37.384	-3.719	20	3	0.468	0.00300	0.154	0.255	2.904
	Hueznar	HUE	37.933	-5.697	20	2	0.100	0.00066	0.218	-1.868*	0.998
	Arreo	ALA	42.778	-2.991	20	2	0.479	0.00315	0.239	2.024	5.159
	Elorz	EL	42.798	-1.667	19	4	0.585	0.00275	0.105	-0.921	1.200
	Expo	EXP	41.671	-0.909	13	3	0.615	0.00405	0.200	1.009	3.086
	Gijón	GIJ	43.536	-5.640	15	3	0.257	0.00085	0.121	-1.317	-0.379
	Jiloca	JIL	40.544	-1.293	15	2	0.419	0.00207	0.210	1.078	3.248
	Leza	LEZ	42.441	-2.311	20	2	0.268	0.00177	0.134	-0.138	3.143
	Almenara	ALM	39.761	-0.183	20	1					
	Brugent	BRU	42.006	2.607	20	2	0.337	0.00222	0.168	0.565	3.843
	Ecomuseu	ECO	40.724	0.722	20	3	0.195	0.00099	0.159	-2.056**	0.136
	Alpedrete	MAD	40.667	-4.016	20	2	0.442	0.00073	0.221	1.026	1.169
	Júcar	ALB	39.148	-1.809	11	3	0.618	0.00114	0.192	0.036	-0.113
	Mundo	MUN	38.458	-1.761	20	1					
	Sa Pobla	SAP	39.791	3.063	5	2	0.400	0.00263	0.400	-1.094”	2.202
	Soller	SOL	39.787	2.794	5	3	0.700	0.00428	0.205	0.562	1.090
	Carucedo	CAR	42.488	-6.784	5	2	0.400	0.00132	0.400	-0.973	1.040
	Chozas	CHO	42.518	-5.714	4	2	0.500	0.00247	0.433	-0.754	1.716
	Valparaiso	VAL	41.995	-6.288	2	2	1.000	0.01151	0.500	0.000	1.946
	Pisuerga	VLB	41.801	-4.588	21	3	0.343	0.00172	0.105	-0.742	1.384
Portugal					114	4	0.399	0.00118			
	Aboboda	ABO	38.736	-9.319	15	2	0.343	0.00169	0.171	0.342	2.710
	Lousal	LOU	38.027	-8.431	20	2	0.479	0.00079	0.239	1.262	1.311

	Alpiarça	POR	39.245	-8.594	20	4	0.642	0.00291	0.123	-0.727	1.429
	R. de Monsaraz	REG	38.478	-7.522	20	2	0.505	0.00083	0.253	1.430	1.409
	Requeixo	REQ	40.592	-8.526	20	2	0.100	0.00016	0.218	-1.164	-0.879”
	Vila-Rica	VILA	41.229	-7.096	19	1					
France					84	5	0.561	0.00218			
	Marais Bruges	BOR	44.903	0.596	20	1					
	Briere	BRI	47.343	-2.246	20	1					
	Tour du Valat	CAM	43.508	4.668	20	2	0.526	0.00346	0.263	2.511	5.567
	Lamartine	TOU	43.506	1.341	21	2	0.095	0.00016	0.213	-1.164	-0.919”
	Rochechevreux	*FR1*	*45.467*	*1.217*	*1*	*1*					
	Rochechevreux	*FR2*	*46.467*	*1.217*	*1*	*1*					
	Givrezac	*FR3*	*45.403*	*0.216*	*1*	*1*					
Italy					60	6	0.731	0.00266			
	Bernate	BER	45.485	8.795	20	2	0.479	0.00079	0.239	1.262	1.311
	Monterotondo	LAZ	42.052	12.547	20	2	0.505	0.00083	0.253	1.430	1.409
	Fucecchio	TOS	43.810	10.794	20	5	0.716	0.00448	0.150	0.257	1.608
Holland	Hardinxveld-Giess	HOL	51.817	4.836	20	1					
Belgium					19	2	0.409	0.00067			
	Bioul	BIO	50.339	4.809	12	1					
	Ecaussinnes	ECA	50.576	4.139	7	2	0.476	0.00078	0.238	0.559	0.589
United Kingdom	Hampstead-Heath	LON	51.561	-0.162	20	1					
ASIA											
Japan					122	4	0.476	0.00237			
	Wakamatsu	FUK	33.911	130.782	20	1					
	Hourai	HOK	42.939	143.224	20	1					
	Waga	IWA	39.436	140.776	9	2	0.556	0.00274	0.278	1.948	3.276
	Ohfuna	KAN	35.353	139.529	20	2	0.521	0.00257	0.261	2.266	4.362
	Rakusho	OKA	34.714	133.933	20	3	0.279	0.00106	0.108	-0.626	0.286
	Ohtsu	SHI	35.013	135.865	10	2	0.533	0.00263	0.267	1.831	3.338
	Kanda	TOK	35.685	139.774	13	3	0.410	0.00240	0.164	-1.335”	1.625
	Saitama	*SA*	*35.850*	*139.650*	*10*	*2*	*0.533*	*0.00263*	0.267	1.831	3.338
China					293	2	0.350	0.00173			
	Shanghai	*SH*	*31.030*	*121.230*	*8*	*2*	*0.571*	*0.00282*	0.286	1.982	3.149
	Jiaxing	*JX*	*30.750*	*120.770*	*10*	*2*	*0.356*	*0.00175*	0.178	0.021	2.334
	Binhu. Wuxi	*WXB*	*31.520*	*120.280*	*7*	*2*	*0.571*	*0.00282*	0.286	1.811	2.920
	Nantong	*NT*	*32.020*	*120.870*	*7*	*2*	*0.286*	*0.00141*	0.350	-1.358”	1.514
	Xiaba village	*XB*	*32.200*	*118.870*	*8*	*2*	*0.571*	*0.00282*	0.286	1.981	3.149

Wuxi	*WX*	*31.570*	*120.300*	*8*	*2*	*0.536*	*0.00264*	0.268	1.601	2.988
Wangjiang	*WJ*	*30.120*	*116.700*	*8*	*1*					
Maanshan	*MAS*	*31.550*	*118.500*	*10*	*2*	*0.356*	*0.00175*	0.178	0.021	2.338
Chaohu	*CH*	*31.620*	*117.870*	*8*	*2*	*0.429*	*0.00211*	0.214	0.458	2.469
Hefei	*HF*	*31.820*	*117.230*	*7*	*2*	*0.286*	*0.00141*	0.350	-1.358”	1.514
Dingyuan	*DY*	*32.280*	*117.830*	*10*	*2*	*0.556*	*0.00274*	0.278	2.057	3.451
Nanbei Port	*NBP*	*29.720*	*116.170*	*8*	*2*	*0.250*	*0.00123*	0.331	-1.448”	1.415
Zhongxian	*ZX*	*30.280*	*108.030*	*10*	*2*	*0.533*	*0.00263*	0.267	1.831	3.338
Jianyang	*JY*	*30.380*	*104.550*	*10*	*2*	*0.467*	*0.00230*	0.233	1.152	2.985
Chongqing	*CQS*	*29.550*	*106.530*	*6*	*1*					
Ningbo	*NB*	*29.880*	*121.550*	*7*	*1*					
Xuyi-culture	*XYC*	*33.000*	*118.500*	*7*	*1*					
Xuyi-wild	*XYW*	*33.030*	*118.420*	*10*	*1*					
Xiaguan district	*XG*	*32.080*	*118.750*	*10*	*1*					
Baguazhou township	*BGT*	*32.170*	*118.820*	*8*	*1*					
Guangfengwei	*CJR*	*30.120*	*116.870*	*8*	*1*					
Sanli township	*SLT*	*29.750*	*116.220*	*8*	*1*					
Poyang lake	*PYL*	*28.870*	*116.430*	*10*	*1*					
Youlan. Nanchang	*NCYL*	*28.520*	*116.120*	*6*	*1*					
Nanhu lake	*NHL*	*30.020*	*114.030*	*8*	*1*					
Yuni Lake	*YNL*	*30.000*	*112.200*	*7*	*1*					
Xiantao	*XT*	*30.300*	*113.400*	*8*	*1*					
Qianjiang	*QJ*	*30.400*	*112.600*	*10*	*1*					
Liangzi lake	*LZL*	*30.000*	*114.000*	*10*	*1*					
Honghu lake	*HLL*	*29.700*	*113.400*	*8*	*1*					
Changhu lake	*CHL*	*30.300*	*112.100*	*6*	*1*					
Yuanjiang	*YJ*	*28.850*	*112.370*	*10*	*1*					
Ningxiang	*NX*	*28.280*	*112.550*	*8*	*1*					
Dongting lake	*DTL*	*29.300*	*113.020*	*10*	*1*					
Dongting Lakeside	*DTLs*	*29.350*	*113.130*	*9*	*1*					

Number of sequences (N), number of haplotypes (*h*), haplotype diversity (Hd), nucleotide diversity (π).

871 Table 2. Analysis of Molecular Variance (AMOVA) within the native area of *Procambarus clarkii*,
872 giving corresponding values for F_{CT} (difference among groups), F_{SC} (differences among localities
873 within groups), and F_{ST} (differences among all localities). Five groups were considered: the native
874 localities in Mexico, Texas, east Louisiana, west Louisiana and upstream Mississippi River.

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation
México - Texas – E. Louisiana – W. Louisiana – Upstream Mississippi River				
Among groups	4	77.228	0.53574	29.04 (F_{CT} = 0.290, p = 0.000)
Among localities within groups	15	39.135	0.17294	9.38 (F_{SC} = 0.132, p = 0.002)
Within localities	157	178.350	1.13598	61.58 (F_{ST} = 0.384, p = 0.000)
Total	176	294.712	1.84466	

875

876

877 Table 3. Analysis of Molecular Variance (AMOVA) among the native and introduced localities of
878 *Procambarus clarkii* worldwide and among the five zones (native range, west Americas, east USA,
879 Europe and Asia), listing the corresponding values for F_{CT} (difference among groups), F_{SC}
880 (differences among localities within groups), and F_{ST} (differences among all localities)

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation
Native Area – Invaded Area				
Among groups	1	69.842	0.19760	14.35 (F_{CT} = 0.143, p = 0.000)
Among localities within groups	114	932.781	0.63021	45.76 (F_{SC} = 0.534, p = 0.000)
Within localities	1293	710.207	0.54927	39.89 (F_{ST} = 0.601, p = 0.000)
Total	1408	1712.829	1.37708	
Native Area – West Americas – East USA – Europe – Asia				
Among groups	4	491.791	0.49942	36.04 (F_{CT} = 0.360, p = 0.000)
Among localities within groups	111	510.831	0.33716	24.33 (F_{SC} = 0.380, p = 0.000)
Within localities	1293	710.207	0.54927	39.63 (F_{ST} = 0.604, p = 0.000)
Total	1408	1712.829	1.38585	

881

Table 4. Analysis of Molecular Variance (AMOVA) within Europe between northern and southern distribution of *Procambarus clarkii*, listing the corresponding values for F_{CT} (difference among groups), F_{SC} (differences among localities within groups), and F_{ST} (differences among all localities).

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation
North (UK, HOL, BIO, ECA, BRI) – **South European distribution** (rest of European localities)				
Among groups	1	59.646	0.40773	40.74 (F_{CT} = 0.407, p = 0.000)
Among localities within groups	37	101.955	0.13481	13.47 (F_{SC} = 0.227, p = 0.000)
Within localities	628	287.734	0.45817	45.78 (F_{ST} = 0.542, p = 0.000)
Total	666	449.334	1.00071	

vii. Figure captions

Figure 1. Haplotype frequencies of *Procambarus clarkii* in the 122 localities distributed worldwide. The size of pie charts is proportional to the sample size. Haplotypes restricted to one sampling locality (i.e., private haplotypes) are coloured in white within pie charts, while haplotypes shared between localities are shaded using colours. Black spots show each one of the 122 localities used, and dark grey areas represent the native range of *Procambarus clarkii*. a) Global map; b) United States and Mexico; c) Hawaiian Islands; d) Costa Rica; e) close-up of Louisiana (US) within its native range; f) East Asia (China and Japan) and; g) Europe.

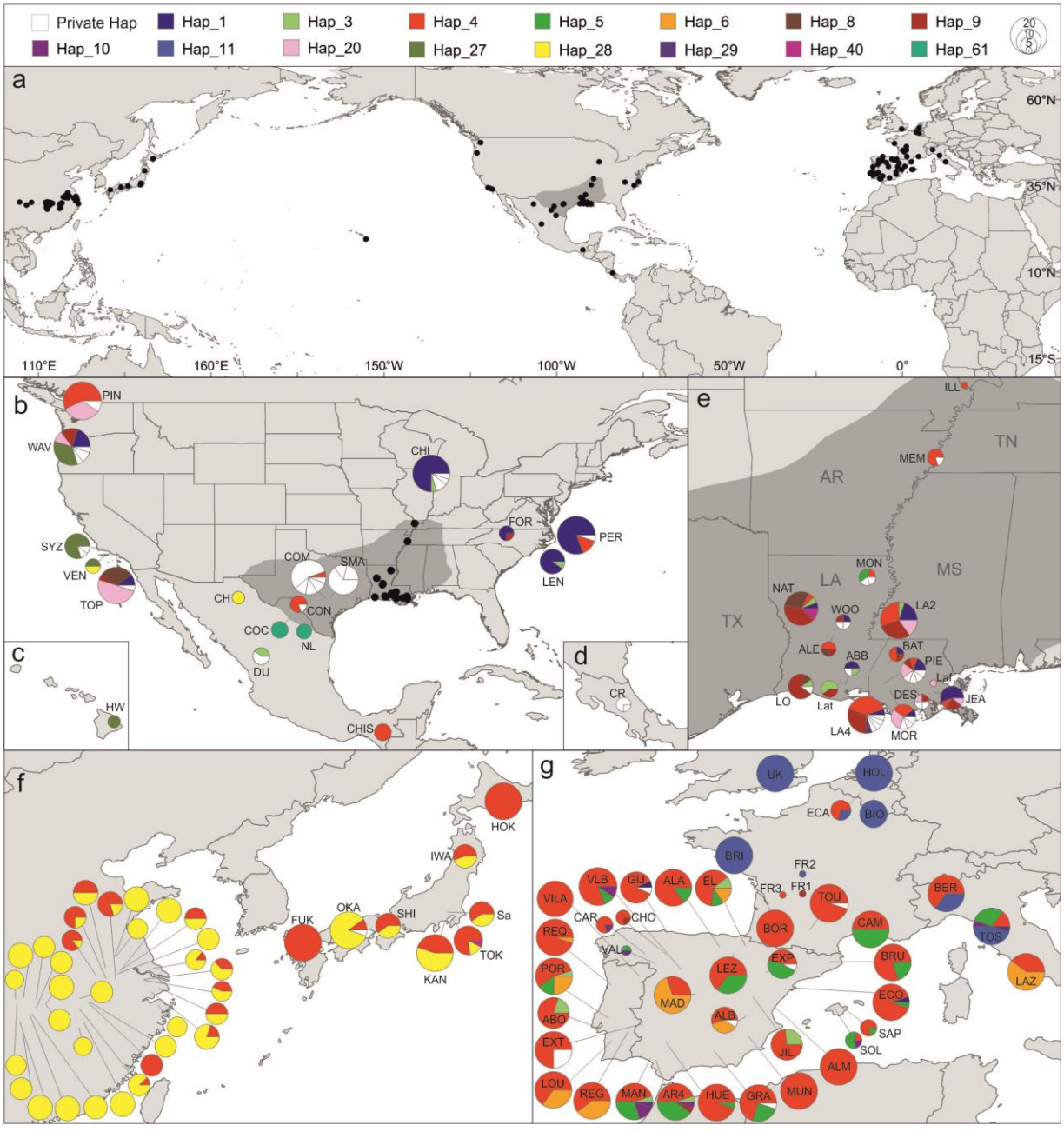


Figure 2. Haplotype network (statistical parsimony-based) for cytochrome c oxidase subunit I (COI) sequences of the red swamp crayfish, *Procambarus clarkii*. Each circle represents one haplotype and its size is proportional to the haplotype frequency. Within the network, each line between haplotypes represents a mutational change and small black dots show unsampled haplotypes inferred from the data. Localities from the same geographical region share the same colour. Haplotypes with non-synonymous changes are indicated by *.

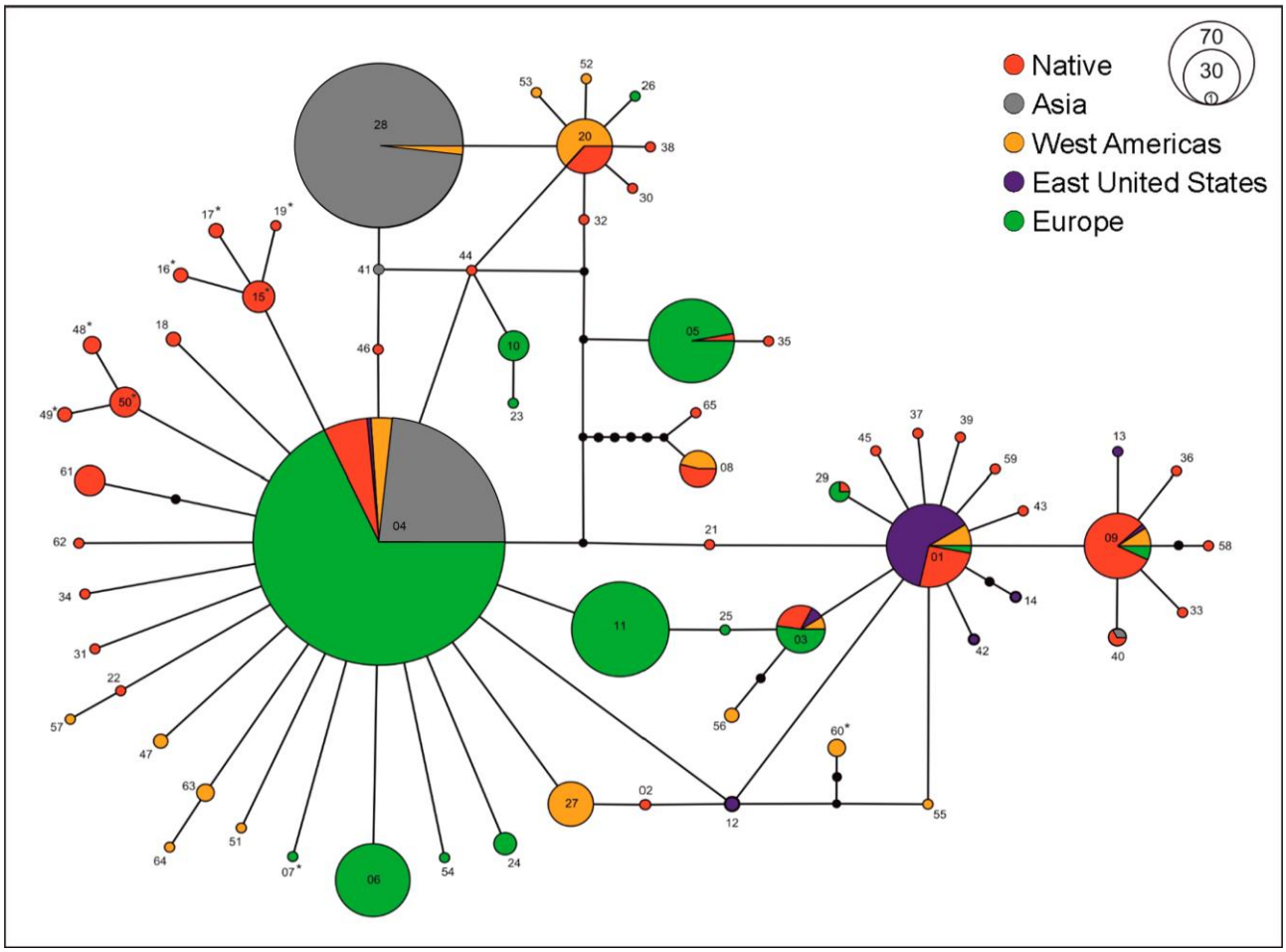


Figure 3. NMDS analysis on D_{est} Jost distances. The graph depicts the pairwise dissimilarity between localities in a low-dimensional space where each point represents one population, ellipses depict established groups and dashed ellipses their 95% confidence intervals (CI). For a better interpretation, a green triangle indicates overlapping Central European localities (BIO, LON, BRI and HOL), a black "X" indicates the Mexican and Chinese overlapping localities (CHt, NB, XYc, XYw, XG, BGt, CJr, SLt, PYL, NCyL, NHL, YNL, XT, QJ, LZL, HLL, CHL, YJ, NX, DTL, DTLs) and a black star indicates the European, Asian and Mexican overlapping localities (BOR, VILA, MUN, ALM, WJ, CQs, HOK, FUK, CHIS).

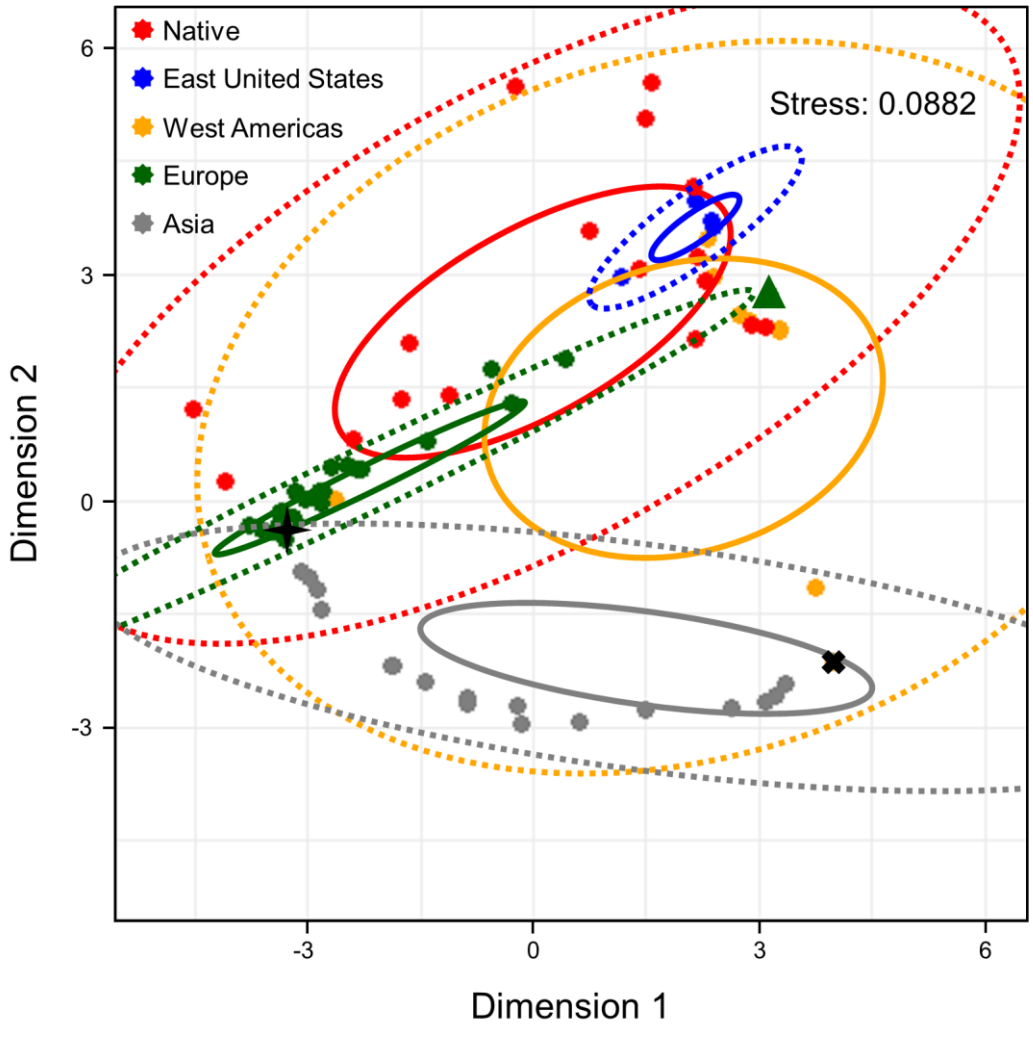
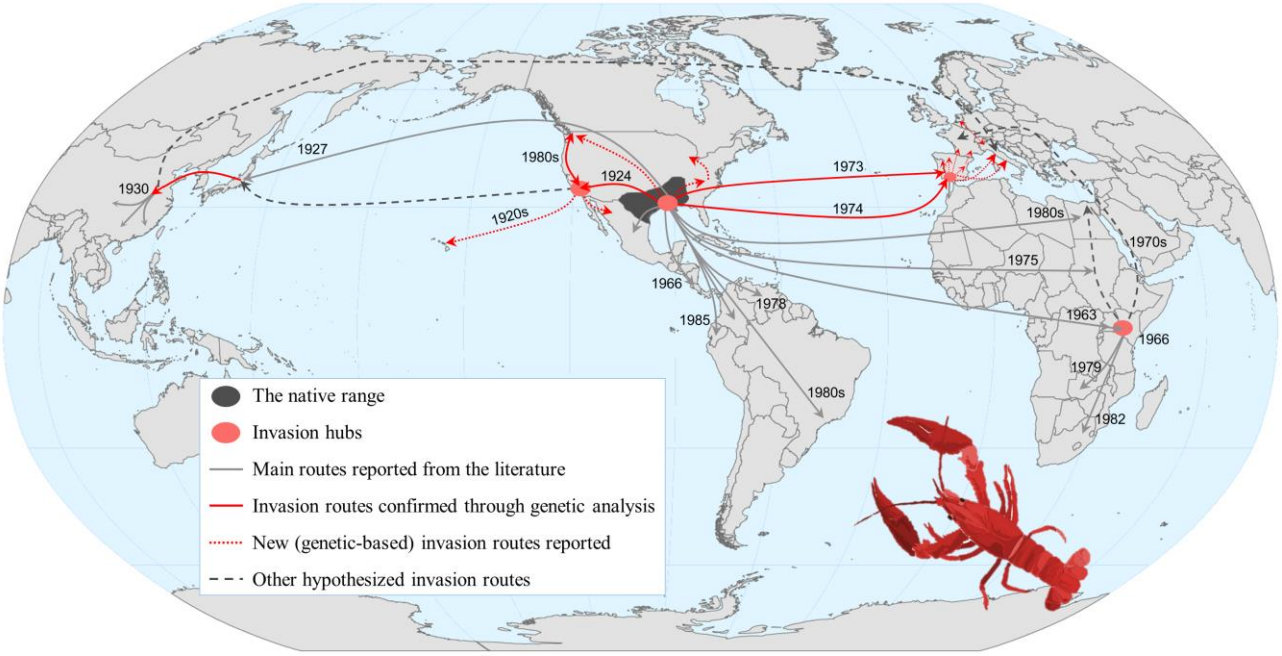


Figure 4. The global invasion routes of the red swamp crayfish, *Procambarus clarkii*, native from southern US and northeastern Mexico, based on mtDNA (present study) and reports from the literature. Main and secondary introduction routes are confirmed, described and hypothesized. Relevant invasion hubs, which usually act as recipients and sources of new invasions, are shown as red circles: Louisiana (in the native range), California, Kenya and Spain.



List of legends of supplementary material

Table S1. List of haplotypes used for this study and their synonyms for the 608 bp COI gene fragment used. GenBank accession numbers in bold were obtained from Almerão et al. (2018), and those in italics were obtained from (Li et al., 2012). A same GenBank accession number can appear in more than one of our haplotypes due to different number of base pairs in the alignment. * indicates that the other study found the haplotype in this zone but was not detected in our study.

Table S2. Haplotype frequencies grouped by the 5 biogeographical zones. Bold type indicates haplotypes shared between two localities at least.

Table S3 Pairwise ϕ_{ST} values (below diagonal) and pairwise Jost's D_{est} values with confidence intervals (above diagonal) for the mtDNA COI of *Procambarus clarkii*. Both significant p- values of ϕ_{ST} following a FDR correction and confidence intervals of D_{est} not enclosing 0, are represented in bold. Locality names are abbreviated as in Table 1.

Figure S1. TCS haplotype network for cytochrome c oxidase subunit I (COI) sequences of the red swamp crayfish, *Procambarus clarkii,* in the native range. Each circle represents one haplotype and its size is proportional to the haplotype frequency. Within the network, each line between haplotypes represents a mutational change and small black dots show unsampled haplotypes inferred from the data. Localities from the same location share the same colour.